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A METHOD FOR TESTING MICROORGANISMS ON SOLID SURFACE AND KIT THEREFOR

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A METHOD FOR TESTING MICROORGANISMS ON SOLID SURFACE AND KIT THEREFOR

[Kotaihyomennobiseibutsujikkenho oyobi sonotamenokitto]

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Claims

1. A method for testing microorganisms present in a test specimen, using a microorganism test pressure-sensitive adhesive sheet having an pressure-sensitive adhesive layer comprising a mainly water-insoluble polymeric compound, with pressing to and peeling from the test specimen surface of the pressure-sensitive adhesive layer to accumulate microorganisms, followed by detection of microorganisms by contacting the pressure-sensitive adhesive layer surface with an aqueous solution containing one or more of colorizing materials that stain the microorganisms.

2. The method described in Claim 1, characterized in that the aqueous solution containing colorizing material is not filtered.

3. The method described in Claim 1 or 2, wherein the colorizing material is fluorescent material.

4. The method described in any of Claims 1-3, wherein the pressure-sensitive adhesive layer surface of the microorganism test pressure-sensitive adhesive sheet has a surface smoothness of below 20 μm.

5. A microorganism test kit comprising a microorganism test pressure-sensitive adhesive sheet having a pressure-sensitive adhesive layer comprising mainly a water-insoluble polymeric compound, and an aqueous solution containing one or more colorizing materials that can stain microorganisms.

6. The kit described in Claim 5, wherein the colorizing material is fluorescent material.

7. The kit described in Claim 5 or 6, wherein the pressure-sensitive adhesive layer surface of the microorganism test pressure-sensitive adhesive sheet has a surface smoothness of below
20 μm.

Detailed explanation of the invention

[0001]

Technological field of the invention

The present invention concerns a novel method for testing microorganisms, more specifically concerns a microorganism testing method involving collection of microorganisms using a pressure-sensitive adhesive layer and detection of the collected microorganism by staining the microorganisms.

[0002]

Prior art

Conventionally, for observation and counting of microorganisms such as bacteria, etc., that can not be observed by visual observation of a test specimen surface, an incubation method is used wherein a solid plate medium formed from agar, etc., is pressed against the specimen surface for transfer of the microorganisms from the specimen surface to the plate medium, followed by incubation of the plate medium under a desired condition then measuring the resulting colonies visually or under a microscope. Such methods include, e.g., agar stamp method using Food Stamp (product of Nissui Seiyaku Co.)

[0003]

In the membrane method using a membrane filter, etc., capable of capturing microorganisms, microorganisms are washed out of specimen surface by rinsing with saline

solution, phosphate buffer solution, etc., sufficiently, then the washings are filtered through a membrane filter to collect microorganisms on the membrane filter, the microorganisms are contacted sufficiently with a liquid medium for formation of colonies on the filter, and the colonies are counted. Also, this membrane method can be used for microorganism detection without incubation by staining the microorganisms collected on the filter, followed by counting microorganisms under a microscope, etc.

[0004]

However, in agar stamp method, etc., the stamp can be used only once on one specimen surface, and the collection efficiency varies according to the water content of agar medium, thus often reproducibility is poor and microorganism collection efficiency may not be sufficient. Also, commonly, in the incubation method, contamination may occur between microorganisms, and pure incubation is not possible due to interaction between microorganisms on the medium, leading to inaccurate evaluation. In addition, the incubation method is limited to live microorganisms, leading to problems of inaccurate detection. Furthermore, the incubation method requires one or two or more days of incubation time, thus it has a serious restriction for real time microorganism monitoring.

[0005]

On the other hand, in the membrane method, while liquid specimens such as aqueous solutions can be filtered directly, non-liquid specimens require sampling by cotton swab and washing for collection of microorganism, which requires a significant effort, and washing and filtering may results in swelling of collected materials other than microorganisms, making observation and measurement difficult.

[0006]

More recently, technology for detecting ATP (adenosine triphosphate) inside microorganism cells has been developed. However, even this is limited to microorganisms dispersed in water, and collecting microorganisms is still problematic.

[0007]

Problems to be solved by the invention

Thus, it is an object of the present invention to provide a novel microorganism testing method free from the drawbacks of the conventional methods described above, especially to provide a microorganism testing method enabling simple real time monitoring of the presence

and/or number of microorganisms on a solid surface. It is another object of the present invention to provide a microorganism testing kit that can be used in such a method.

[0008]

Means to solve the problems

As a result of an intense study in order to achieve the objectives, we have attained the present invention using a microorganism test pressure-sensitive adhesive sheet having an pressure-sensitive adhesive layer comprising a mainly water-insoluble polymeric compound, with pressing to and peeling from the test specimen surface of the pressure-sensitive adhesive layer to accumulate microorganisms, followed by detection of microorganisms by contacting the pressure-sensitive adhesive layer surface with an aqueous solution containing one or more colorizing materials that stain the microorganisms. Thus, microorganisms on solid surfaces can be detected rapidly and simply.

[0009]

Namely, the present invention concerns a method for testing microorganisms present in a test specimen, using a microorganism test pressure-sensitive adhesive sheet having an pressure-sensitive adhesive layer comprising a mainly water-insoluble polymeric compound, with pressing to and peeling from the test specimen surface of the pressure-sensitive adhesive layer to accumulate microorganisms, followed by detection of microorganisms by contacting the pressure-sensitive adhesive layer surface with an aqueous solution containing one or more of colorizing materials that stain the microorganisms.

[0010]

In the method of the present invention, the microorganisms captured and accumulated on the surface of the pressure-sensitive adhesive layer of the pressure-sensitive adhesive sheet (also referred to pressure-sensitive adhesive surface hereafter) are stained as they are retained on the pressure-sensitive adhesive surface without incubation, thus microorganisms such as bacteria, fungi, virus, etc., can be detected and/or counted in real time by visual observation or observation of the coloration state and color intensity using microscopes or other optical devices.

[0011]

Also, the present invention provides a microorganism testing kit suitable of carrying out the microorganism testing method of the present invention simply and rapidly. Namely, another aspect of the present invention is a microorganism testing kit comprising a microorganism test pressure-sensitive adhesive sheet having a pressure-sensitive adhesive layer comprising a mainly

water-insoluble polymeric compound, and an aqueous solution containing one or more colorizing materials that can stain microorganisms.

[0012]

Practical embodiment of the invention

The pressure sensitive adhesive sheet for the microorganism test used in the present invention is formed by laminating a water-insoluble polymeric compound-based pressure-sensitive adhesive layer on a substrate. Such pressure-sensitive adhesive layer has sufficient tackiness to capture the microorganisms on the specimen surface and a smoother surface structure while the adhesive is not dissolved even when immersed in the aqueous solution for microorganism staining.

[0013]

A backing layer may be installed on the opposite side of the adhesive layer of the adhesive sheet for reinforcing substrate strength and for forming background color for enhanced recognition of the microorganisms stained with the colorizing material and for prevention of optical scattering.

[0014]

The substrate of the pressure-sensitive adhesive sheet for the microorganism test is a water-insoluble flexible material so that the pressure-sensitive adhesive surface does not form large undulations and can be adhered freely on bent and narrow areas. The substrates may be, but are not limited to, polyesters, polyethylene, polyurethane, vinyl chloride, fabrics, nonwoven fabrics, paper, polyethylene-laminated paper, etc. Among these, smooth polyester, polyethylene, vinyl chloride and polyurethane are desirable. While there are no specific limitations on the substrate support as long as sufficient strength is provided, a thickness of about 5-200 μm is preferred.

[0015]

The pressure-sensitive adhesive should have tackiness sufficient for capturing the microorganisms on the specimen surface and is not soluble in water. Such adhesives may be, but are not limited to, polymeric compounds such as natural rubber, synthetic rubber, silicone pressure-sensitive adhesive, polyacrylic acid-based polymeric compound, polyacrylate salt-based polymeric material, ethylene-acetic acid [sic] copolymer, polyvinyl ether, polyvinyl ester, etc. Two or more of these may be used together. Such polymeric compounds can be prepared by known methods. For example, polyacrylic acid-based polymeric compounds can be prepared by

solution polymerization, emulsion polymerization, suspension polymerization, bulk polymerization, photochemical polymerization, etc. If needed, the polymeric compounds may be compounded with usual additives such as tackifying agents, antioxidants, crosslinking agents, fillers, etc.

[0016]

In counting captured microorganisms using a microscope, etc., for focusing on the microorganisms collected on the pressure-sensitive adhesive layer surface, the surface smoothness (undulation) should be below 20 μm. With the surface smoothness below 20 μm, the microscope focusing range is expanded, making the counting easier. The surface smoothness can be obtained by using a surface coarseness measuring device, or cross section of the pressure-sensitive adhesive sheet for the microorganism test is observed through an electron microscope, etc., and the undulation height on the pressure-sensitive adhesive layer surface is measured.

[0017]

The pressure-sensitive adhesive sheet for microorganism test used in the present invention can be prepared by known methods. For example, a solution of a polymeric compound used for the pressure-sensitive adhesive layer is coated on a substrate and dried at a temperature from room temperature to 200°C, or a calendering, casting, extrusion method, etc., can be used. The resulting sheets are cut into desired shapes and used.

[0018]

In the present invention, the pressure-sensitive adhesive sheets for microorganism test may be irradiated with radiation such as electron beam, γ ray, etc., for sterilization and crosslinking of the polymeric compounds used for the pressure-sensitive adhesive layer. The sterilization may be also done by ethylene oxide gas, and by sealing the sterilized product in a microorganism-shielding packaging material, the products can be stored in sterile conditions.

[0019]

Next, the microorganism testing method of the present invention is explained. The microorganisms to be tested may be prokaryotes such as bacteria, actinomyces, etc., eukaryotes such as yeast, mildew, etc., algae, virus, cultured animal and plant cells.

[0020]

The pressure-sensitive adhesive sheets for microorganism test may be pressed against floor, wall, etc., for efficient transfer and collection of microorganisms on the specimen surface. For a test surface considered to have relatively low microorganism content, collection may be done by repeated pressing of the same adhesive surface to the same area. In the method of the present invention, incubation of agar stamp method is not needed, thus there is no concern about colony contamination or phase changes during incubation, thus multiple microorganism collection is possible. Thus, by repeated pressing, a large number of microorganisms can be collected as in membrane filter method involving filtration and concentration of microorganisms dispersed in water.

[0021]

Next, the pressure-sensitive adhesive sheet containing the collected microorganism is cut into a desired size, and the area with collected microorganisms is immersed into an aqueous solution containing colorizing material for microorganism staining. If excessive colorizing material has to be removed, the microorganism-collected area may be washed with sterile water. If the microorganism-collected area has to be dried after staining of microorganisms, the drying may be done by air drying, natural drying, vacuum drying, etc. In microscope observation, when the colorizing material is a fluorescent dye, the microscope observation is done under stimulating light, imparting a detection function, and thus microorganisms can be counted directly. According to the present invention, since incubation is not needed, microorganisms can be detected within a few minutes.

[0022]

Any colorizing material that develops color upon action with the cellular components of the microorganisms to be tested can be used in the present invention, e.g., fluorescent dye solutions that stain nucleic acids and proteins. The colorizing dyes may be fluorescent nucleic acid salt analogs, fluorescent dyes staining nucleic acids, dye solutions staining proteins, environmental fluorescent probes used for structural analysis of proteins, dye solutions used for analysis of cell membrane and membrane potential, dye solutions used for labeling fluorescent antibodies, etc., in the case of general microorganisms; dye solutions developing color by cell respiration, etc., in the case of aerobic bacteria; dye solution staining mitochondria, dye solution staining golgiosome, dye solution staining vesicle, dye solution reacting or modifying esterase in cells, etc., in the case of eucaryotic microorganisms; dye solution used in observation of bone tissue, dye solutions used for nerve cell tracers, etc., in the case of higher animal cells. These can be observed under a fluorescence microscope.

[0023]

With proper selection of the type of the such coloring materials, counting entire microorganisms, staining and counting of only microorganisms with respiration activity, staining and counting of only microorganisms having esterase activity, staining and counting of certain strains and genus by multiple staining using multiple number of colorizing materials can be used over wide ranging areas.

[0024]

Microorganism detection and counting may be done visually or by forming optical images using an optical microscope, florescence microscope, laser microscope or other optical device and then analyzing the images. The other optical devices may be, e.g., laser scanning site meter involving high-speed scanning of microorganisms with laser light and graphical display of signals obtained from each microorganism. With no need for incubation, in the present invention, the microorganisms on the pressure sensitive adhesive surface of the pressure-sensitive adhesive sheet can be detected within several minutes to several tens of minutes.

[0025]

The present invention is also to provide a microorganism testing kit using the above microorganism testing method. The above microorganism testing kit used in the above microorganism testing method comprises a microorganism test pressure-sensitive adhesive sheet having a pressure-sensitive adhesive layer comprising mainly water-insoluble polymeric compound, and an aqueous solution containing one or more colorizing materials that can stain microorganisms. The microorganism testing sheet and coloring materials are similar to those illustrated for the method of the present invention.

[0026]

In an example of application of the present invention, the pressure-sensitive adhesive face is adhered to the test face for transfer of microorganisms present on the test face, then the microorganisms are stained without incubation with observation of single cells of the microorganisms. Thus, this method can be used for environmental inspection with rapid measurement of the cleanliness of the specimen. Furthermore, with recovery at the single cell level, microorganisms can be collected by repeated contact of the pressure-sensitive adhesive sheet to the specimen face and concentrated. Application fields can be extended into environmental microorganism inspection at medical and food sites.

[0027]

Examples

Next, the present invention is explained in detail with examples. However, these examples are merely for illustration purposes and do not limit the present invention in any way.

[0028]

Application Example 1

1) Preparation of pressure-sensitive adhesive sheet for microorganism test

2-Ethylhexyl acrylate	70 parts by weight
Ethoxyethyl acrylate	25 parts by weight
Acrylic acid	5 parts by weight
Ethyl acetate	150 parts by weight
Azoisobutyronitrile [sic]	0.3 part by weight

In a polymerization reactor, the above ingredients were stirred while the reactor was purged with nitrogen and polymerized by heating at 55-65°C for about 10 h. Then, the content was further stirred at 70°C for about 2 h. The resulting copolymer had glass transition temperature 212°C and gel fraction 31.5%. Next, this pressure-sensitive adhesive was coated to a dry thickness of 20 μm on a silicone-treated release paper and dried at 130°C for 5 min. The adhesive side was adhered to a corona-treated 75 μm-thick polyester film and cut to an effective adhesive area of about 10 cm^2.

[0029]

2) Measurement

4 μL of aseptic phosphate buffer solution-diluted staphylococcus culture was dropped on the surface of plastic and dried naturally to obtain a specimen. The pressure-sensitive adhesive sheet was freed from the release paper and repeatedly pressed against and peeled from the specimen three times for collection of the staphylococcus. The bacteria-collected pressure-sensitive adhesive face was treated with SYBR Green II (SYBR Green II RNA Gel Stain, product of Molecular Probes Inc.) diluted 10,000-fold with sterilized deionized water by dropwise addition, dried naturally, and number of the green-stained bacteria was measured by using a fluorescence microscope (x1000) of 490 nm stimulation wavelength. Results are given in Table together with results of Comparative Example 1. Bacteria detection was comparable to or better than the wiped membrane filter method.

[0030]

Comparative Example 1

For the comparative examples of the present invention, wiped membrane filter method was carried out. Specimen prepared similarly from culture as Application Examples 1 and 2 was wiped using sterilized water-containing cotton swap (tradename: Fukifukicheck, product of Eikenkizai) and suspended in sterilized saline solution, followed by staining for 15 min with addition of SYBR Green II diluted 10,000-fold, filtering through a polycarbonate filter of pore diameter 0.2 μm. The stained bacteria collected on the filter was dried naturally and counted similarly as in application examples. Results are given in Table 1 together with Application Example 1.

[0031]

Table 1

被験物	供試菌数	粘着シート（実施例1）	拭き取りーメンブレンフィルター法（比較例1）
プラスチック表面	6.0×10^{6}cells	3.2×10^{6}cells	2.0×10^{6}cells
木製机表面	未計測	1.8×10^{6}cells/cm^2	2.5×10^{6}cells/cm^2
プラスチック表面	未計測	1.3×10^{6}cells/cm^2	4.5×10^{6}cells/cm^2

Key: 1 Specimen
2 Number of cells used
3 Pressure-sensitive adhesive sheet (Application Example 1)
4 Wiped membrane plate method (Comparative Example 1)
5 Plastic surface
6 Wood desk surface
7 Not counted
8 Plastic surface

[0032]

Application Example 2

Microorganisms were measured in natural environment for specimens around body without intentional addition of microorganism. Microorganisms were collected, stained and measured similarly as in Application Examples 1 and 2. Results are given in Table 2. Measurement was comparable to or above the wipe membrane filter method.

[0033]

Comparative Example 2

The specimens of Application Example 2 were wiped for collection of microorganisms from specimen surfaces similarly as in Comparative Example 1, and the microorganisms collected were stained, filtered and measured. Results are given in Table 2. While bacteria counts are not shown, in the wiped membrane filter method, hand dirt swelled by water is also collected and shows up in the measurement.

[0034]

Table 2

被験物	粘着シート（実施例2）	拭き取りーメンブレンフィルター法（比較例2）
ロッカーの金属扉	1.4×10^{5}cells/cm^2	3.0×10^{5}cells/cm^2
クーラーボックスの蓋	6.0×10^{5}cells/cm^2	4.0×10^{5}cells/cm^2
木製ドア	1.2×10^{5}cells/cm^2	9.5×10^{5}cells/cm^2

Key: 1 Specimen
2 Pressure-sensitive adhesive sheet (Application Example 2)
3 Wiped membrane filter method (Comparative Example 2)
4 Metal door of locker
5 Cooler box lid
6 Wooden door

[0035]

Application Example 3

According to the present invention, live bacteria were counted for staphylococcus. Staphylococcus on the surface of specimen prepared similarly as in Application Examples 1 and 2 were collected on the pressure-sensitive adhesive sheet. Next, the collected bacteria on the pressure-sensitive adhesive sheet was treated with 150 μg/mL 6-carboxyfluorescein diacetate (hereafter referred to 6CFDA, product of Sigma Co.) by dropwise addition, dried naturally, and counted for the number of bacteria emitting a green color under a fluorescence microscope similarly as in Application Example 1 and 2). The results are given in Table 3 together with the number of bacteria stained with SYBR Green II as in Application Example 1. Bacteria with esterase activity were detected in ten and several minutes, amounting to about 60-95% of total number of bacteria detected with STBR Green II.

[0036]

Table 3

被験物	供試菌数	6CFDA染色	STBR Green II染色
プラスチック表面	5.0×10^{5}cells	2.0×10^{5}cells	3.2×10^{5}cells
プラスチック表面	8.4×10^{5}cells	1.0×10^{5}cells	1.3×10^{5}cells
プラスチック表面	4.6×10^{5}cells	8.0×10^{4}cells	9.4×10^{4}cells

Key: 1 Specimen
2 Number of cells used
3 6CFDA staining
4 STBR Green II staining
5 Plastic surface

[0037]

Effect of the invention

According to the present invention, without incubation and a washing-filtering process, microorganisms can be collected efficiently from a solid surface, thus microorganisms can be collected, detected and/or counted conveniently in real time.